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Comparison of RT-PCR With Other Diagnostic Assays for Rapid Detection of Influenza Viruses

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To compare the effectiveness of reverse transcription-polymerase chain reaction (RT-PCR), shell vial culture and cytospin assay as laboratory techniques for rapid diagnosis of influenza infections, a retrospective study was carried out on 270 aliquots of oropharyngeal swabs collected from October 1993 to March 1996 and already characterized by standard isolation procedures, and a prospective study in which 65 clinical samples taken from patients with influenza-like syndrome between October 1996 and March 1997 were tested. In the retrospective study, using conventional isolation as the gold standard, the sensitivity of RT-PCR and cytospin assay for virus A was 100% (95% confidence interval (CI), 89.1–100) and for virus B it was 100% (95% CI, 56.1–100) compared with 77.5% (95% CI, 61.1–88.6) and 71.4% (95% CI, 30.3–94.9) for shell vial culture. The specificity of all the three assays was 100% (95% CI, 98.0–100) for virus A and 100% (95% CI, 98.2–100) for virus B. In the prospective study the sensitivity of RT-PCR was greater than that of the other tests considered, both rapid and standard. It is suggested that RT-PCR should be employed in combination with conventional culture techniques in routine diagnosis of influenza infections in order to obtain results more rapidly and to improve virus detection even in circumstances in which standard isolation could be problematic. ***J. Med. Virol. 56: 168–173, 1998.*** © 1998 Wiley-Liss, Inc.

KEY WORDS: influenza detection; rapid assays; RT-PCR

INTRODUCTION

Influenza viruses are a major cause of acute respiratory infection (ARI) and are responsible for high morbidity and mortality worldwide. Although in most patients influenza infection results only in short absences from work or school, there are groups at high risk for which it may constitute a serious illness. During epidemics it can cause 10,000–20,000 excess deaths in the elderly and in patients with chronic cardiovascular and pulmonary diseases [Pachucki, 1992]. The clinical symptoms of influenza are very similar to those associated with other respiratory viruses, often circulating in the community at the same time, so that laboratory confirmation of influenza infection is essential for surveying influenza outbreaks and for assessing the efficacy of vaccines and specific antiviral therapies.

The conventional laboratory diagnosis of influenza is based on virus isolation, either in embryonated chicken eggs or in cell cultures, and subsequent identification of the virus by hemagglutination inhibition or hemadsorption. The problems of these standard reference methods are the time needed (7–20 days for a definite diagnosis) and the difficulty of storing the pathologic specimen correctly in order to preserve viral infectivity. Hence, alternative, rapid diagnostic methods must be developed to enable prompt treatment and prophylaxis with specific antiviral drugs such as amantadine, rimantadine (for influenza A), and neuraminidase inhibitors, since when given within 48 hours of onset of illness, these agents decrease the severity of clinical symptoms [Cherian et al., 1994; Scott and Huang, 1996]. The timely identification of influenza viruses in a community and of new emerging strains would also allow efficient epidemiological surveillance of influenza to be carried out. A variety of techniques involving a molecular biology have been suggested [Zhang and Evans, 1991; Claas et al., 1992; Donofrio et al., 1992; Atmar et al., 1996], or immunocapture ELISA [Chomel, 1989]. The aim of the present study was to optimize laboratory assays for rapid detection of influenza viruses and to evaluate the appropriateness of introducing reverse transcription-polymerase chain reaction (RT-PCR) for routine diagnosis of influenza infections.

MATERIALS AND METHODS

Specimens

This study was divided into two parts: retrospective and prospective.

Retrospective study. Aliquots of original oropharyngeal swabs (270) collected from October 1993 to

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March 1996, stored at −80°C, and characterized by standard isolation were tested. There were 39 positive for A/H3N2, 1 positive for A/H1N1, 7 positive for B, 223 negative for influenza virus but positive for other agents causing ARI (94 positive for RSV, 12 for Parainfluenza, 16 for Adenovirus, 29 for Rhinovirus, 49 for *Str. pneumoniae,* 16 for *Chl. pneumoniae,* and 7 for *Haem. influenzae*). Furthermore, the complete antigenic characterization of positive specimens was available, carried out by the World Health Organization (WHO) Reference Center of London, which identified the type, subtype (for influenza A), and viral strain.

Prospective study. Oropharyngeal swabs (65) collected from patients with influenza-like syndrome between October 1996 and March 1997 were also tested. These two groups of specimens were tested by shell vial culture [Espy et al., 1986], and the cytospin assay, and RT-PCR [Atmar et al., 1996]; all tests were run in duplicate.

Specimen Handling and Preparation

After collection, pharyngeal swabs were broken into a single vial containing transport medium (tryptose broth of pH 7–7.2 supplemented with 0.5% of gelatin or seroalbumin) and transported on ice to the laboratory. Each swab was vortexed, extracted, pressing it against the vial, and charged with a mixture of antibiotics, with a final concentration of 20,000 U/ml of penicillin and 20,000 μg/ml of streptomycin. After incubation at 4°C for 1 hour, specimens were centrifuged at 1,500 × g for 15 minutes at 4 °C. Supernatants were tested immediately or divided into several aliquots stored at −80°C until examination.

Shell Vial Assay

Specimens were inoculated in 200 μl aliquots into shell vial monolayers of Madin-Darby canine kidney (MDCK) cells washed previously with phosphate-buffered saline (PBS) supplemented with the above mixture of antibiotics to prevent virus neutralization by non specific serum inhibitors. The samples were then placed in vials, centrifuged at 700 × g for 1 hour at 35°C [Seno et al., 1990; Engler and Selepak, 1994; Marcante et al., 1996; Reina et al., 1996] and decanted; serum-free basal medium Eagle (BME), with 1 μg/ml of trypsin was added before incubation at 35°C. After 20–24 hours, each vial was washed with PBS, fixed in cold acetone-methanol (2:1) for 10 minutes, and stained finally with type-specific monoclonal antibodies directed against virus nucleoprotein and conjugated to fluorescein for direct immunofluorescence assay.

Cytospin Assay

Specimens were inoculated in 200 μl aliquots into tube monolayers of MDCK cells that had been washed with PBS and charged with the above mixture of antibiotics. The samples were placed in tubes and incubated at 35°C for 1 hour, then, 2 ml of serum-free maintenance buffer, according to Meguro et al.[1979]: BME, supplemented with 5 ml/l of antibiotics, 0.1% bovine serum albumin, sodium bicarbonate 2%, and 1% trypsin, was put in each tube before the final incubation at 35°C. Three to four days after inoculation, without waiting for the appearance of a visible cytopathic effect, BME was decanted and 2.5% trypsin solution added before incubation at 35°C for 20 minutes to remove MDCK cells from the culture tubes. The cells were suspended in 1 ml of PBS and 200 μl of this suspension was cytocentrifuged at 500 rpm for 5 minutes at room temperature. After drying, the slides were fixed with cold acetone for 10 minutes and stained with type-specific monoclonal antibodies (see above).

RT-PCR

For the RT-PCR analysis, sequential steps were employed. First, we determined the possible positivity of specimens and simultaneously the virus type using primers targeted to the matrix protein gene (segment 7). Subsequently, for the type A viruses detected, in parallel we determined subtype specificity of viral surface antigens, haemagglutinin, and neuraminidase, using primers specific for viral segments 4 and 6.

A nested PCR (two-step amplifications using two pairs of primers, outer and inner) was always carried out to obtain the best analytical sensitivity and specificity [Clementi et al., 1993; Severini et al., 1993]. To prevent carryover, all precautions were undertaken to minimize this problem according to Kwok [1990] and Sambrook et al.[1989].

Oligonucleotide Primers

The oligonucleotide primers used in the different steps, for both virus detection/typing and subtyping, have been published previously [Zhang and Evans, 1991; Donofrio et al., 1992]. They were all used at a final concentration of 10 pmol/μl.

RNA Extraction and Reverse Transcription

Viral RNA was extracted from a 200 μl volume of oropharyngeal swabs using the acid guanidinium and phenol method of Chomczynski and Sacchi [1987].

cDNA complementary to viral RNA was prepared using Moloney murine leukemia virus (M-MLV) reverse transcriptase: 15 μl of extracted RNA was mixed with 7 μl of 5 × first strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$), 1.5 μl of random hexanucleotides (dNTPs 200 μM), 1 μl of RNase inhibitor (40 IU/μl), 1 μl of M-MLV reverse transcriptase (200 IU/μl), 3.5 μl of DTT (100 mM), and 7 μl of oligonucleotide; the reaction was carried out in a thermal cycler at 42°C for 60 minutes, followed by 15 minutes at 90°C. Primers used in parallel on each sample were AMPDII for virus type A and BMPDII for type B.

PCR Amplification

PCR mixtures contained 27 μl of sterile deionized water, 5 μl of 10 × buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, 10% triton X-100), 1 μl of dNTPs (200 μM), 0.5 μl of DNA-Taq polymerase (2 IU/μl), 5 μl of each outer oligonucleotide and 6 μl of target cDNA.

cDNA was subjected to 34 PCR cycles (94°C × 1 minute, 55°C × 1 minute, 72°C × 1.5 minutes) plus 1 cycle (94°C × 1 minute, 55°C × 1 minute, 72°C × 8 minutes) in a thermal cycler. Outer primers used in this step were AMPDII + AMPA for type A and BMPDII + BMPA for type B. An aliquot (5 μl) of first amplification products underwent a further PCR containing inner primers. PCR mixtures, temperatures and times for this second step were the same as for the first step. The inner primers used were A2 + A1 for type A and BMPCII + BMPB for type B. 10 μl of PCR products were analyzed by electrophoresis on 3% agarose gel (Nusieve:Seakem 5: 1) in 1 × Tris-borate-EDTA (TBE) buffer (89 mM Tris-HCl, 2.5 mM disodium EDTA, 89 mM boric acid). Ethidium bromide (10 mg/ml) was incorporated into both the agarose gel and electrophoresis buffer eliminating a staining step. Amplified products were visualized by ultraviolet transillumination.

Virus A Subtyping

Except for the primers, reverse transcriptase and RT-PCR mixtures were the same as those described above.

Reverse Transcription

Viral haemagglutinin and neuraminidase were subtyped. The primers used for the A/HA region (type A, segment 4) were AH1FII and AH3DII, and for the A/NA region (segment 6) AN1EII and AN2DII. Mixtures were incubated at 42°C × 60 minutes plus 15 minutes at 90°C.

PCR Amplification

cDNA was first subjected to 25 PCR cycles in a reaction containing outer primers. In addition, an aliquot (5 μl) of first amplification products underwent 25 other PCR cycles with inner primers. All amplifications always used a denaturation step at 94°C × 1 minute and a polymerization step at 72°C × 3 minutes, followed by an 8-minute extension at the end of cycling. Specific annealing conditions were adopted for the different primers based on the results of previous experimental trials on virus stock suspensions with a known subtype (50°C for first PCR except for N2 with 52°C; 55°C for nested PCR segment 4 and 52°C or 48°C, respectively, for segment 6 subtype N1 or N2). These conditions provided efficient amplification with no background. The outer primers for the A/HA region were AH1FII + AH1A and AH3DII + AH3A and, for the A/NA region, AN1EII + AN1A and AN2DII + AN2A. The inner primers for the A/HA region were AH1EII + AH1B and AH3CII + AH3B and for the A/NA region, AN1DII + AN1B and AN2CII + AN2B; 10 μl of PCR products were analyzed by electrophoresis on 1.5% agarose gel (Nusieve:Seakem 5:1) in 1 × TBE buffer (89 mM Tris-HCl, 2.5 mM disodium EDTA, 89 mM boric acid).

Sensitivity

The sensitivity of RT-PCR and cytospin assays were compared by a limiting dilution experiment [Severini et al., 1993; Marcante et al., 1996]. Serial 10-fold dilutions of two virus stock suspensions (A/Singapore/6/86/H1N1, with a known titre equivalent to 5,000 hemagglutination units/ml (HU/ml), and B/Yamagata/5/89, with a titre of 160 HU/ml) were prepared in triplicate and each dilution was inoculated into two tube monolayers of MDCK cells (for cytospin assay) and was tested singly at RT-PCR for segment 7.

Furthermore, the limit of sensitivity of RT-PCR was determined in terms of the least quantity of viral RNA detectable by the test: RNA of a virus stock suspension (A/Singapore/6/86/H1N1) was extracted and the amount of RNA was determined by ultraviolet spectrophotometry at 260 nm [Sambrook et al., 1999; Class et al., 1992]. Serial 10-fold dilutions of these RNA solutions were assessed by RT-PCR for the matrix protein gene. Once the highest 10-fold dilution positive at RT-PCR was determined, serial twofold dilutions were prepared, starting from the one before the last still positive 10-fold dilution, for RT-PCR assays to obtain a more accurate evaluation of the test's sensitivity.

Statistical Methods

The 95% confidence intervals (CI) of estimated values were calculated by Fleiss' method.

RESULTS

Retrospective Study

This first analysis tested the sensitivity and specificity of three alternative assays for rapid detection of influenza viruses compared to conventional procedures of viral isolation, based on a collection of oropharyngeal swabs already characterised by standard isolation alone. Table I shows the overall results of the samples analysed. We observed complete agreement between RT-PCR and cytospin assay, for both influenza A and B (14.8% and 2.6% respectively). In contrast, for both viral types, discrepancies between these two methods and shell vial culture were found for both viral types, with the former two recognizing 11 more samples as positive than the latter: nine for influenza A and two for influenza B viruses. The sensitivity, specificity, positive and negative predictive values, and efficiency, of RT-PCR, cytospin assay and shell vial culture are shown in Table II, with conventional isolation as the gold standard. The sensitivity of shell vial culture was 77.5% for influenza A and 71.4% for influenza B compared with the 100% sensitivity of RT-PCR and cytospin assay. The specificity of all the methods was 100%.

Prospective Study

This confirmed the effectiveness of RT-PCR, which proved more sensitive than all culture methods for influenza A. The overall results of the samples analysed are reported also in Table I. We found no discordance for the only pharyngeal swab positive for influenza B, whereas two samples were positive only at RT-PCR. The five samples positive for influenza A (matrix protein gene) at RT-PCR were characterised subsequently as subtype H3N2 by RT-PCR for two other genomic

TABLE I. Total Results of Influenza A and B Detection in Oropharyngeal Samples by Cytospin Assay, Shell Vial Culture, and RT-PCR

N° samples	Cytospin assay	Shell vial culture	RT-PCR
Retrospective study (samples = 270)			
	Influenza A positivity		
31	+	+	+
9	+	–	+
230	–	–	–
% Positive	14.80%	11.50%	14.80%
	Influenza B positivity		
5	+	+	+
2	+	–	+
263	–	–	–
% Positive	2.60%	1.80%	2.60%
Prospective study (samples = 65)			
	Influenza A positivity		
3	+	+	+
2	–	–	+
60	–	–	–
% Positive	4.62%	4.62%	7.69%
	Influenza B positivity		
1	+	+	+
64	–	–	–
% Positive	1.54%	1.54%	1.54%

regions. The two samples that were positive only by RT-PCR were negative for other pathogenic agents causing ARI; moreover, in one case the presence of influenza infection was confirmed by seroconversion as blood samples were available and showed a serological response to influenza A haemagglutinin (subtype H3), although virus was not isolated from respiratory secretions. The 59 negative swabs for influenza were: 11 positive for RSV, 5 for Parainfluenza, 7 for Adenovirus, 9 for Rhinovirus, 4 for *Str. Pneumoniae,* 2 for *Haem. influenzae,* and 21 negative for all agents examined/ Table III shows the sensitivity and specificity of the various techniques, singly and combined.

Sensitivity Threshold

Comparison of the sensitivity threshold of RT-PCR and cytospin assay, using serial 10-fold dilutions of two stock virus suspensions, proved the superiority of RT-PCR, an amplification product was clearly visible up to the titre corresponding to 5×10^{-3} HU/ml for type A, and 1.6×10^{-2} HU/ml for type B. Cytospin assay detected as positive the virus A suspensions that contained 5×10^{-2} HU/ml (only 1 of the 6 tube monolayers in which we showed this dilution gave a positive result) and the virus B stock suspensions containing 1.6 HU/ml.

The determination of the RT-PCR sensitivity threshold, in terms of the least quantity of viral RNA detectable, showed positivity in dilutions with as little as 1 pg of virus nucleic acid.

DISCUSSION

Influenza transmission is controlled selectively by vaccinating high-risk groups [CDC, 1990], timely detection of the first cases in a community, and adoption of control measures allow to avoid the spread of infection in institutions and nursing homes [Staynor et al., 1994; Gomolin et al., 1995]. Moreover, specific antiviral drugs are effective in prophylaxis of influenza and also in treatment if administered early during the acute phase of illness [Delker et al., 1980; Wiselka, 1994]. Unfortunately, attaining these goals and a prompt and rational application of antiviral therapies have often been hampered due to the lack of standard rapid laboratory assays for the rapid diagnosis of influenza infections and detection of new emerging viral strains. The rapid diagnosis systems based on immunoassay can be applied only to freshly collected specimens and does not allow diagnosis in frozen samples or after delayed examination of specimens [Chomel, 1989; Donofrio et al., 1992]. These tests will always suffer from the requirement that the antigen of interest must be reasonably intact or it will not bind with antibody [Zhang and Evans, 1991]. Thus samples that have been subjected to storage under nonideal conditions [Zhang and Evans, 1991; Donofrio et al., 1992], or recovered from partially degraded specimens may not retain labile antigenic determinants and certainly will not retain viable virus. Under these circumstances, methods capable of detecting viral nucleic acids rather than protein offer particular advantages since both DNA and RNA are considerably more stable than protein [Golenberg et al., 1990].

Recently developed molecular biology techniques are of great potential interest as they can detect small amounts of viral nucleic acid in pathological specimens rapidly and provide amplified DNA suitable for further molecular analysis. In this study we assessed the possibility of introducing RT-PCR as a reference method for routine diagnosis of influenza infection.

The retrospective analysis showed that the performance of RT-PCR, cytospin assay, and conventional virus isolation was the same, whereas shell vial cultures had a lower sensitivity but the same specificity. Furthermore, with RT-PCR it was possible to subtype A viruses rapidly without using specific antisera against viral strains of current epidemiological reference, usually prepared in chickens and in ferrets. This was confirmed by the results of the RT-PCR assays of the 40 known virus A-positive samples of the retrospective study. Moreover, the intentional inclusion in the retrospective study of a large number of specimens negative for influenza virus but positive for other pathogens causing ARI allowed us to compare the specificity of the three methods considered; no false positives were found in the 223 samples that were known to be negative for influenza. A further proof of the high specificity of the techniques was the results obtained with the negative reagent controls, always included in all the experiments.

The prospective study confirmed the effectiveness of RT-PCR [Ellis et al., 1997], which proved more sensitive not only more than the other two assays of rapid detection of influenza infections but also more than conventional virus isolation: two virus A positive speci-

TABLE II. Comparison of Three Rapid Tests With Virus Isolation as the Gold Standard in the Retrospective Study

Assay	% Sensitivity (95% CI)	% Specificity (95% CI)	% PPV[a] (95% CI)	% NPV[b] (95% CI)	% Efficiency (95% CI)
		Influenza A			
RT-PCR	100(89.1–100)	100(98.0–100)	100(89.1–100)	100(97.9–100)	100(98.2–100)
Cytospin	100(89.1–100)	100(98.0–100)	100(89.1–100)	100(97.9–100)	100(98.2–100)
Shell vial	77.5(61.1–88.6)	100(98.0–100)	100(86.3–100)	96.2(92.7–98.1)	96.7(93.6–98.4)
		Influenza B			
RT-PCR	100(56.1–100)	100(98.2–100)	100(56.1–100)	100(98.2–100)	100(98.2–100)
Cytospin	100(56.1–100)	100(98.2–100)	100(56.1–100)	100(98.2–100)	100(98.2–100)
Shell vial	71.4(30.3–94.9)	100(98.2–100)	100(46.3–100)	99.2(97.0–99.9)	99.3(97.1–99.9)

[a]Positive predictive value.
[b]Negative predictive value.

TABLE III. Comparison of Sensitivity and Specificity of RT-PCR, Shell Vial Culture, and Cytospin Assay for Influenza A in the Prospective Study

Reference	Shell vial	Cytospin assay	RT-PCR
		% Sensitivity (95% CI)	
Shell vial (n = 3)	–	100 (31.0–100)	100 (31.0–100)
Cytospin (n = 3)	100 (31.0–100)	–	100 (31.0–100)
RT-PCR (n = 5)	60 (17.0–92.7)	60 (17.0–92.7)	–
Standard isolation (n = 3)	100 (31.0–100)	100 (31.0–100)	100 (31.0–100)
All[a] (n = 5)	60 (17.0–92.7)	60 (17.0–92.7)	100 (46.3–100)
		% Specificity (95% CI)	
Shell vial (n = 3)	–	100 (92.7–100)	96.8 (87.8–99.4)
Cytospin (n = 3)	100 (92.7–100)	–	96.8 (87.8–99.4)
RT-PCR (n = 5)	100 (92.5–100)	100 (92.5–100)	–
Standard isolation (n = 3)	100 (92.7–100)	100 (92.7–100)	96.8 (87.8–99.4)
All[a] (n = 5)	100 (92.5–100)	100 (92.5–100)	100 (92.5–100)

[a]Positive results by shell vial culture, cytospin assay, RT-PCR, standard isolation, and all methods are used for comparison.

mens at RT-PCR grew neither in MDCK cells nor in embryonated chicken eggs. Circumstances in which RT-PCR can detect viral nucleic acid even when virus isolation by standard assay systems was impossible have been reported often [Paton et al., 1992; Cherian et al., 1994]. Culture assays need a minimum number of virus particles with infecting power in the clinical samples [Cherian et al., 1994; Gomolin et al., 1995], and infecting power is often lost between collection of specimens and execution of laboratory tests due to storage under nonideal conditions. In these circumstances, assays able to detect viral nucleic acid, without requiring viable viruses, would offer a great advantage especially when they have to be performed on samples taken from patients with influenza-like syndrome 3–4 days after the sudden beginning of illness. Indeed, in these cases viral isolation in cell culture or in embryonated chicken eggs is impossible because of decreasing viral charge and virus inactivation [Class et al., 1993; Cherian et al., 1994].

The high sensitivity and specificity of RT-PCR in both the retrospective and prospective analyses demonstrate the effectiveness of a nucleic acid amplification technique as an alternative test for rapid diagnosis of influenza infection. However, its introduction for routine diagnosis will require final validation in a larger number of clinical specimens and also a definitive standardization to enable it to be used in public health laboratories, leaving conventional culture and embryonated chicken eggs to WHO reference centres. The use of RT-PCR as a reference assay for influenza virus detection should allow efficient epidemiological surveillance and a prompt, rational administration of antiviral therapies based on the immediate identification of patients with influenza infections. If all important respiratory pathogens can be detected reliably by PCR, simultaneous detection in the same small aliquot of sample will be very useful. PCR may become a useful tool in respiratory surveillance.

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